The unfolded protein response is shaped by the NMD pathway

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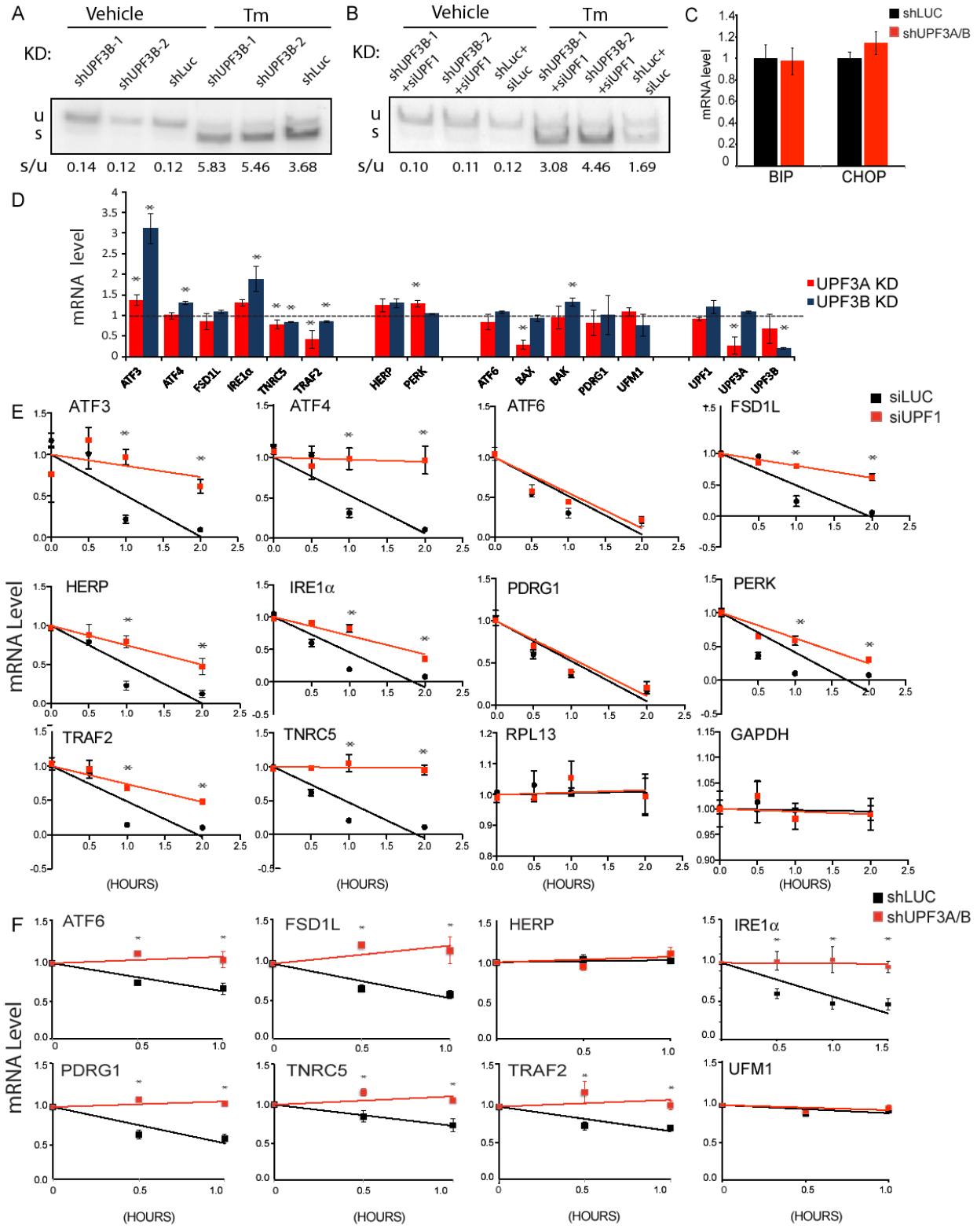
Supplementary Information:

- **Supplementary Figure S1. Associated with Figure 1.**
- **Supplementary Table S1 and S2. Associated with Figure 1.**
- **Supplementary Figure S2. Associated with Figure 2.**
- **Supplementary Figure S3 and S4. Associated with Figure 4.**
- **Supplementary Table S3. General information of primary sequences.**

Figure S1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Vehicle
Tm
KD:
shUPF3B-1
shUPF3B-2
shLuc
u
s
s/u
0.14 0.12 0.12 5.83 5.46 3.68
B
Vehicle
Tm
KD:
shUPF3B-1 +siUPF1
shUPF3B-2 +siUPF1
shLuc+ siLuc
shUPF3B-1 +siUPF1
shUPF3B-2 +siUPF1
shLuc+ siLuc
s/u
0.10 0.11 0.12 3.08 4.46 1.69
C
shLUC
shUPF3A/B
mRNA level
BIP
CHOP
D
mRNA level
UPF3A KD
UPF3B KD
ATF3
ATF4
FSD1L
IRE1α
TNRC5
TRAF2
HERP
PERK
ATF6
BAX
BAK
PDRG1
UFM1
UPF1
UPF3A
UPF3B
E
siLUC
siUPF1
ATF3
ATF4
ATF6
FSD1L
mRNA Level
HERP
IRE1α
PDRG1
PERK
TRAF2
TNRC5
RPL13
GAPDH
(HOURS)
F
shLUC
shUPF3A/B
mRNA Level
ATF6
FSD1L
HERP
IRE1α
PDRG1
TNRC5
TRAF2
UFM1
(HOURS)

Supplementary Figure S1. IRE1 signaling and half-lives of UPR component mRNAs in NMD-deficient cells.

A,B [γ32P]-end-labeled RT-PCR analysis of unspliced (u) and spliced (s) *XBP1* mRNA levels in HeLa cell clones stably depleted of UPF3B. shUPF3B-1 and -2 are independent UPF3B-depleted cell clones and shLUC cells are negative-control cells stably transfected with a construct expressing a shRNA against luciferase. HeLa dells were also transiently tranfected with a siRNA against the core NMD factor, UPF1, or Luciferase, as indicated. The cells were treated with a high-dose of Tm [2 μg/ml] or vehicle (DMSO) for 4 hrs. The values were normalized with RPL19 mRNA and are the mean (± SEM) (n=6), statistically analyzed by t-test (*P < 0.05).

C qPCR analysis of HeLa cells stably depleted of UPF3A and UPF3B using shRNAs (as previously described [19]). Control HeLa cells are stably transfected with a shRNA luciferase construct (shLUC). The values were normalized with RPL19 mRNA and are the mean (± SEM) (n=6), statistically analyzed by *t-test* (*P < 0.05).

D qPCR analysis of HeLa cells stably depleted of UPF3A or UPF3B using shRNAs (as previously described [19]) or transiently depleted of UPF1 (as in panel B). A value of 1 (doted line) indicates expression in control HeLa cells stably transfected with a shRNA luciferase construct (shLUC). The values were normalized with RPL19 mRNA and are the mean (± SEM) (n=6), statistically analyzed by *t-test* (*P < 0.05).

E,F mRNA half-life analysis of HeLa cells Cells were treated with Actinomycin D to terminate transcription and samples were collected on the indicated time points. The values shown are the average fold change (mean ± SEM) relative to the 0 hr time point (set as "1"). The values were normalized with RPL19 mRNA and are the mean (± SEM) (n=3 for both control and NMD factor-depleted cells), statistically analyzed by *t-test* (*P < 0.05).

Supplementary Table 1: NMD-inducing features in human transcripts encoding selected UPR components.

Transcript	ID number	NMD feature	Reference
ATF3	NM_001030287 NM_001040619 NM_001206484 NM_001206486 NM_001206488 NM_001674	uORF; AS-PTC; Long 3'UTR (1672,1222nt)	Mendell et al. 2004
ATF4	NM_001675 NM_182810	uORF	Mendell et al. 2004
ATF6	NM_007348	Long 3'UTR (5416nt)	
BAK	NM_001188	Long 3'UTR (1257nt)	
BAX	NM_001291428 NM_001291429 NM_001291431 NM_001291430 NM_004324 NM_138761 NM_138763 NM_138764	ND	
FSD1L	NM_001145313 NM_001287191 NM_001287192	Long 3'UTR (5950nt)	
HERP	NM_001010989 NM_001272103 NM_014685	Long 3'UTR (782nt)	
IRE1α (ERN1)	NM_001433	Long 3'UTR (958nt)	
PDRG1	NM_030815	Long 3'UTR (852nt)	
PERK (EIF2AK3)	NM_004836	uORF; Long 3'UTR (995nt)	
TNRC5 (CNPY3)	ENST00000394142 NM_006586	AS-PTC	
TRAF2	NM_021138	uORF; Long 3'UTR (714nt)	
UFM1	NM_001286703 NM_001286704 NM_001286705 NM_016617	Long 3'UTR (2307nt; 2229nt)	

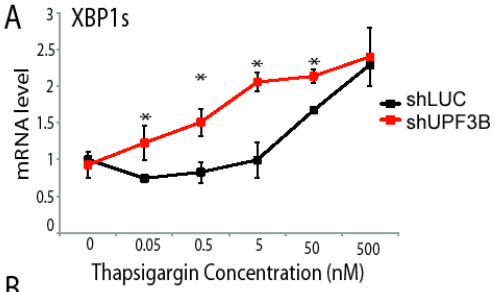
AS-PTC: Alternative spliced isoform harboring a PTC (a stop codon >55 nt upstream of at least one splice junction); uORF: upstream open reading frame; ND: no known NMD-inducing feature detected.

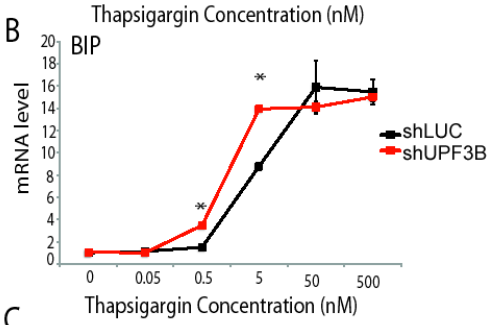
Supplementary Table 2: NMD-inducing features in mouse (*Mus Musculus*) transcripts encoding selected UPR components.

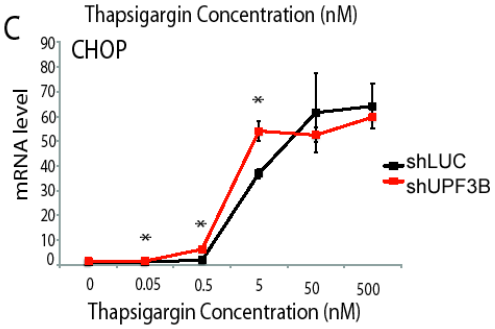
Transcript	ID number	NMD feature	Reference
ATF3	NM_007498	uORF; Long 3'UTR (1185nt)	
ATF4	NM_001287180 NM_009716	uORF	Weischenfeldt et al. 2008
ATF6	NM_001081304	Long 3'UTR (5422nt)	
BAK	NM_007523	uORF; Long 3'UTR (1119nt)	
BAX	NM_007527	uORF;	
FSD1L	NM_001195284 NM_007837	Long 3'UTR (5846nt)	
HERP	NM_022331	ND	
IRE1α (ERN1)	NM_023913	Long 3'UTR (922nt)	
PDRG1	NM_178939	Long 3'UTR (764nt)	Weischenfeldt et al. 2008
PERK (EIF2AK3)	NM_010121	uORF; Long 3'UTR (952nt)	
TNRC5 (CNPY3)	NM_028065	Long 3'UTR (978nt)	
TRAF2	NM_001290413 NM_009422	Long 3'UTR (1428nt)	
UFM1	NM_026435	Long 3'UTR (4552nt)	

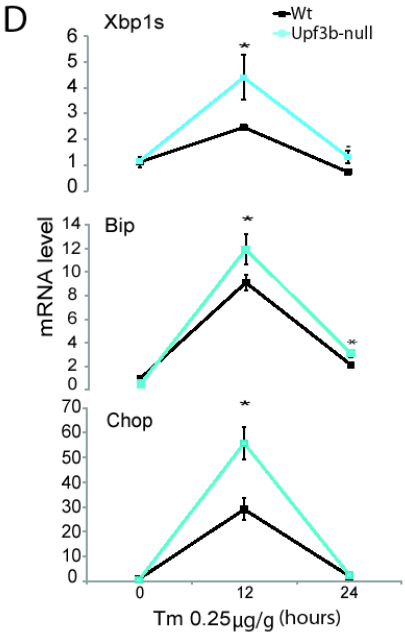
AS-PTC: Alternative spliced isoform harboring a PTC (a stop codon >55 nt upstream of at least one splice junction); uORF: upstream open reading frame; ND: no known NMD-inducing feature detected.

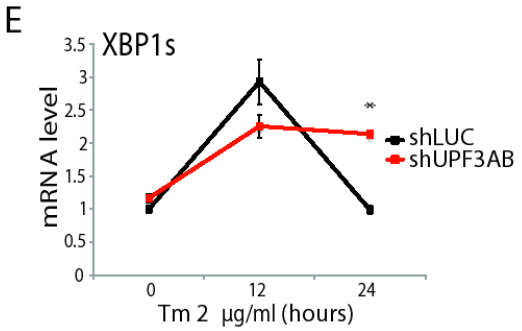
Figure S2

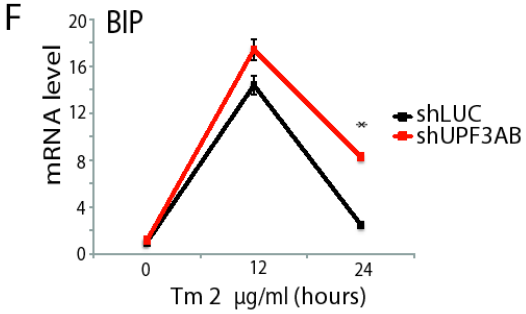

A
XBP1s
mRNA level
shLUC
shUPF3B
Thapsigargin Concentration (nM)

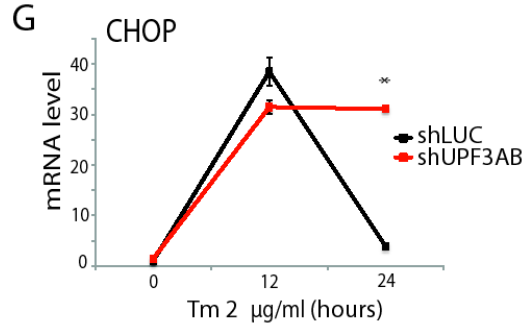

B
BIP
mRNA level
shLUC
shUPF3B
Thapsigargin Concentration (nM)


C
CHOP
mRNA level
shLUC
shUPF3B
Thapsigargin Concentration (nM)


D
Xbp1s
Wt
Upf3b-null
Bip
Chop
mRNA level
Tm 0.25µg/g (hours)


E
XBP1s
mRNA level
shLUC
shUPF3AB
Tm 2 µg/ml (hours)


F
BIP
mRNA level
shLUC
shUPF3AB
Tm 2 µg/ml (hours)


G
CHOP
mRNA level
shLUC
shUPF3AB
Tm 2 µg/ml (hours)

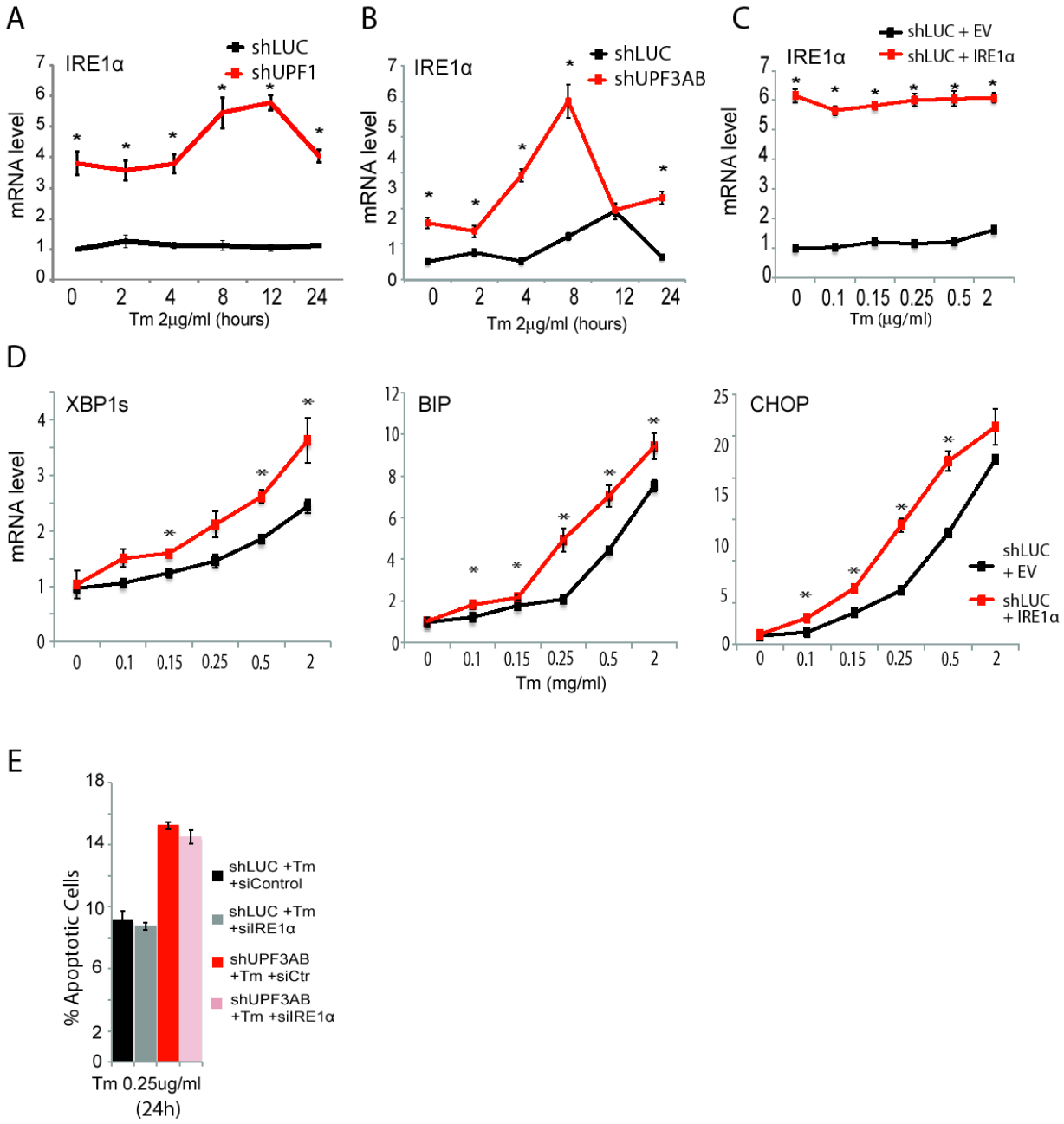
Supplementary Figure S2. NMD raises the UPR activation threshold and promotes UPR attenuation.

A-C qPCR analysis of spliced *XBP1* (*XBP1s*), *BIP*, and *CHOP* mRNAs in HeLa cells stably depleted of the NMD factor UPF3B (shUPF3B) treated with increasing concentrations of Thapsigargin. HeLa cells stably transfected with a construct expressing an shRNA against luciferase (shLUC) serve as a negative control. The values were normalized with RPL19 mRNA and are the mean (± SEM) (n=9), statistically analyzed by *t-test* (*$P < 0.05$).

D qPCR analysis of liver from *Upf3b-null* (n=3) and control littermate (WT) mice (n=3) injected IP with Tm [0.25 µg/g] for the time points indicated. Quantification, error bars, and statistical analysis were performed as in Figure 1. The values were normalized with RPL19 mRNA and are the mean (± SEM), statistically analyzed by *t-test* (*$P < 0.05$).

E-G qPCR analysis of Hela cells stably depleted of the NMD factors UPF3A and UPF3B (shUPF3AB) and incubated with Tm [2 µg /ml] for the time points indicated. HeLa cells stably transfected with a luciferase shRNA construct (shLUC) serve as a negative control. The values were normalized with RPL19 mRNA and are the mean (± SEM) (n=6), statistically analyzed by *t-test* (*$P < 0.05$).

Figure S3


A
IRE1α
shLUC
shUPF1
mRNA level
Tm 2μg/ml (hours)
B
IRE1α
shLUC
shUPF3AB
mRNA level
Tm 2μg/ml (hours)
C
IRE1α
shLUC + EV
shLUC + IRE1α
mRNA level
Tm (μg/ml)
D
XBP1s
mRNA level
BIP
Tm (mg/ml)
CHOP
shLUC + EV
shLUC + IRE1α
E
% Apoptotic Cells
shLUC +Tm +siControl
shLUC +Tm +siIRE1α
shUPF3AB +Tm +siCtr
shUPF3AB +Tm +siIRE1α
Tm 0.25ug/ml (24h)

Supplementary Figure S3. Effect of NMD deficiency on ER stress-induced events.

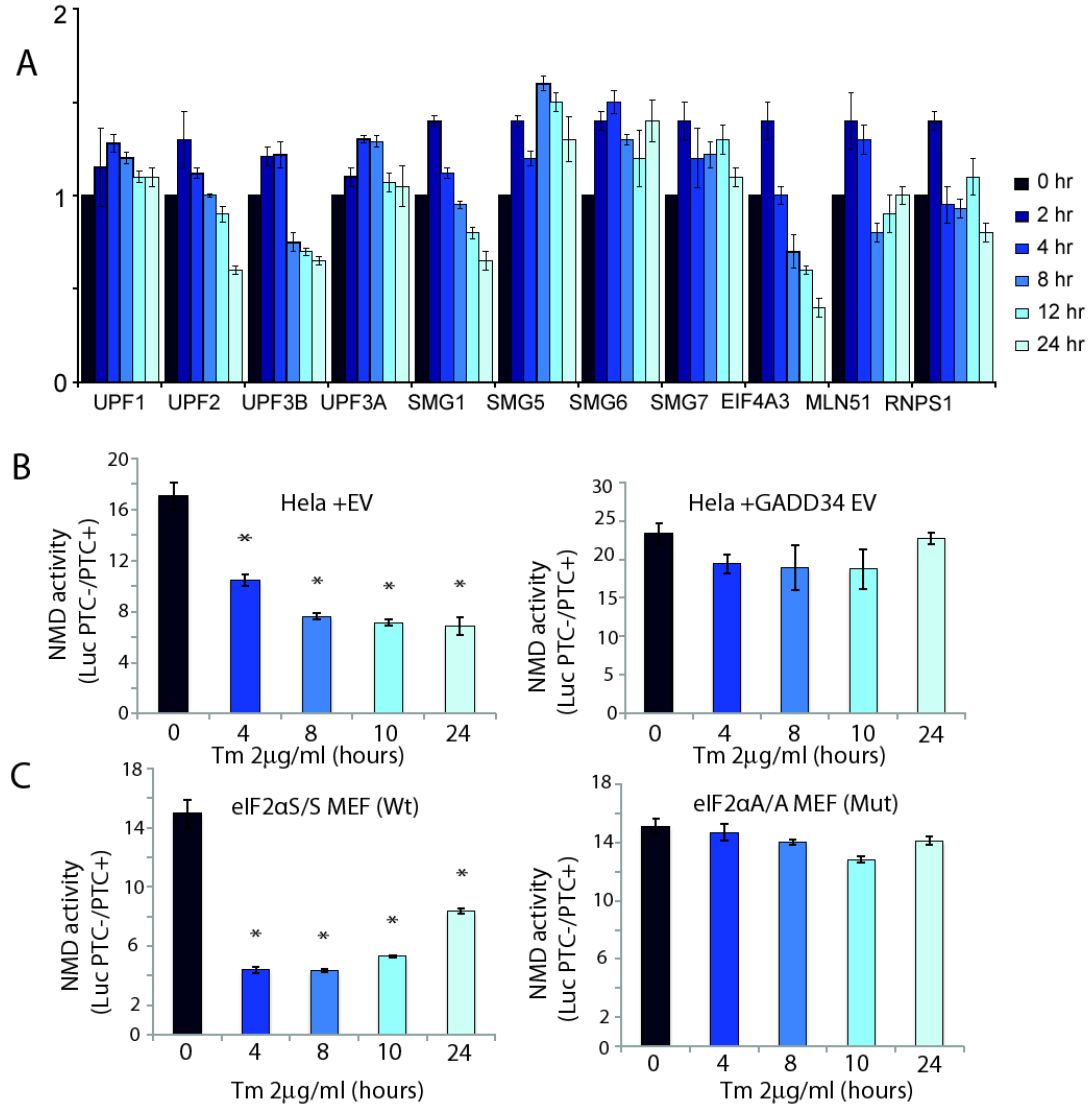
A,B qPCR analysis of HeLa cells (A) depleted of UPF1 (siUPF1) versus control cells (siLUC) (n=3); or (B) stably depleted of UPF3A and UPF3B (UPF3AB) versus control (shLUC) cells treated with a single full dose of tunicamycin [2 μg/ml] for the time points indicated. The values were normalized with RPL19 mRNA and are the mean (± SEM) (n=6), statistically analyzed by *t-test* (*$P < 0.05$).

C qPCR analysis of control HeLa cells (stably transfected with a luciferase shRNA construct shLUC) transiently transfected with 3 ng of either a pCMV14 empty vector (EV) or a pCMV-IRE1 expression vector encoding a NMD-resistant *IRE1* mRNA (NMD-Resist. IRE1). Cells were then treated with incremental doses of Tm for 4 hrs. The values were normalized with RPL19 mRNA and are the mean (± SEM) (n=6), statistically analyzed by *t-test* (*$P < 0.05$).

D qPCR analysis of *XBP1s*, *BIP*, and *CHOP* in the same cells described in panel B. Cells were then treated with incremental doses of Tm for 4 hrs. The values shown in panel B-D are the average (mean ± SEM) from three independent experiments relative to control (shLUC). The values were normalized with RPL19 mRNA and are the mean (± SEM) (n=6), statistically analyzed by *t-test* (*$P < 0.05$).

E FACS analysis indicating the percentage of apoptotic (Annexin-V positive/PI negative) HeLa cells in response to Tm [0.25 μg/ml] treatment for 24 hrs. Shown are HeLa cells stably depleted of the NMD factors UPF3A and UPF3B transfected with an *IRE1*α siRNA (siIRE1) or a control siRNA (siControl) and incubated with Tm [0.25 ug/ml] for 24 hrs. HeLa cells stably transfected with a luciferase shRNA construct (shLUC) serves as a negative control. The values are the mean (± SEM) (n=3), statistically analyzed by *t-test* (*$P < 0.05$).

Figure S4


A
2
1
0
UPF1
UPF2
UPF3B
UPF3A
SMG1
SMG5
SMG6
SMG7
EIF4A3
MLN51
RNPS1
0 hr
2 hr
4 hr
8 hr
12 hr
24 hr
B
Hela +EV
NMD activity
(Luc PTC-/PTC+)
Tm 2µg/ml (hours)
Hela +GADD34 EV
C
eIF2αS/S MEF (Wt)
eIF2αA/A MEF (Mut)

Supplementary Figure S4. Evidence for a NMD-UPR regulatory circuit.

A qPCR analysis of *NMD factors* in HeLa cells treated with full dose of Tm treatment (2 μg/ml) for 24 hrs. The values were normalized with RPL19 mRNA and are the mean (± SEM) (n=3), statistically analyzed by *t-test* (*$P < 0.05$).

B Left panel: NMD activity, as measured by the ratio of luciferase reporter activity in HeLa cells transiently transfected with *Renilla* Luciferase/β-goblin NMD reporter vectors containing either a premature termination codon containing (PTC+) or not (PTC-). These cells were incubated with Tm (2 μg/ml) for the times indicated. The cells were co-transfected with a Firefly luciferase construct (pCI-neo Firefly) to normalize for transfection efficiency. Right panel: NMD activity measured as in the left panel, in HeLa cells co-transfected with a GADD34 expression vector. These cells were incubated with Tm (2 μg/ml) for the times indicated. The values are the mean (± SEM) (n=3), statistically analyzed by *t-test* (*$P < 0.05$).

C Left panel: NMD activity, measured as in the panel E, in wild type eIF2α (eIF2αS/S) MEF cells incubated with Tm (2 μg/ml) for the times indicated. Right panel: NMD activity, measured as in the panel E, in mutated eIF2α (eIF2αA/A) MEF cells incubated with Tm (2 μg/ml) for the times indicated. The values are the mean (± SEM) (n=3), statistically analyzed by *t-test* (*$P < 0.05$).

Supplementary Table S3. Primer sequences.

qPCR primers		Forward	Reverse
IRE1a	human	TGCAGGTCCCAACACATGTGG	TCAGGCCTTCATTATTCTTGC
Ire1a	mouse	GAAACAAGAAACACCACTACCG	GCATATGGAATCACTGGAGGC
PDRG1	human	TGCGCCTCTTACCATATGAC	GGCAGTTCATACTGGGACCT
Pdgr1	mouse	GAAAGGCTGCGGAGTCAACTT	GGGCTGAGGGGATTCAGGTT
ATF3	human	GAGGCGAGCAGAAAGAAATAAG	GTAAGGCTAGAAGGCACTCAC
Atf3	mouse	GCCAAGTGTCGAAACAAGAAAAA	CCTCGATCTGGGCCTTCAG
TRAF2	human	CCAGCATCCTCAGCTCTGGGC	TATCTGGGAAGGCCGAACTGC
ATF4	human	GTCAGTCCCTCCAACAACAGC	GTCATCTATACCCAACAGGGC
Atf4	mouse	ATGGCCGGCTATGGATGAT	CGAAGTCAAACTCTTTCAGATCCATT
FSD1L	human	AGAGTTACAGAGTCAGATTAG	TATATCTAATGACCTTGTTGC
TNRC5	human	GTTCGCCTGCCCAGCAAATGC	TCCTCAAAGGCTGACTTCAGC
CHOP	human	ACCAAGGGAGAACCAGGAAACG	TCACCATTCGGTCAATCAGAGC
Chop	mouse	CTGCCTTTCACCTTGGAGAC	CGTTTCCTGGGGATGAGATA
BAX	human	TCAAGGCTGGCGTGAAATGGC	CACAGGGCCTGTAATCCCAGC
BAK	human	TACCAGCATGGCCTGACTGGC	AGTTCAGGGCTGCCACCCAGC
PERK	human	GAGCAGATTCATGGAAACAGC	GTTAAGGTCCTGACTCTCTCC
XBP1s	human	CCGCAGCAGGTGCAGG	GAGTCAATACCGCCAGAATCCA
XBP1 total	human	GCAAGCGACAGCGCCT	TTTTCAGTTTCCTCCTCAGCG
Xbp1s	mouse	GAGTCCGCAGCAGGTG	GTGTCAGAGTCCATGGGA
Xbp1 total	mouse	AAGAACACGCTTGGGAATGG	ACTCCCCTTGGCCTCCAC
BIP	human	CGGGCAAAGCTGTCAGGAAAG	TTCTGGACGGGCTTCATAGTAGAC
Bip	mouse	CATGGTTCTCACTAAAATGAAAGG	GCTGGTACAGTAACAACTG
HERP	human	CAACAATAACTTACAGGAAGGC	TGAAGACAAGCCATGCTGTGC
UFM1	human	TGGATTCATTCCGGCACCAC	AGGTGTACTTTCAGGAACAACTTT
UPF3A	human	GCGCACGATTACTTCGAGGT	TCAAAACGGTCTCTGAACAGC
Upf3a	mouse	ACCAAAGAGCAGCTGGAA	TTCCAGCTGCTCTTTGGT
UPF3B	human	AGGAGAAACGAGTGACCCTGT	CCTGTTGCGATCCTGCCTA
Upf3b	mouse	AGGAGAAACGAGTGACCCTGT	CCTGTTGCGATCCTGCCTA
RPL19	human	ATGTATCACAGCCTGTACCTG	TTCTTGGTCTCTTCCTCCTTG
Rpl19	mouse	CTGAAGGTCAAAGGGAATGTG	GGACAGAGTCTTGATGATCTC
18S	human	GGACACGGACAGGATTGACA	ACCCACGGAATCGAGAAAGA
G997-1320 F	Reporter	TGGCTGGTGTGGCTAATGC	
G1320 R	Reporter	TCTAATTGTGGTGGCCAGGC	
G997 R	Reporter	CAGCTCAGGGATGACCTTGC	

Cloning primers			
IRE1 3UTR-1	human	CTGGTCACCACAATTAGAGC	GTCAGCACTGTCCTCTGTG
IRE1 3UTR-2	human	AGGGTCACCGTGTGCTTCATG	CAAGAAAGCTTCAAGTTTAGC
IRE1 3UTR-3	human	CTTAGTGTATTGAGCTAGGC	CTCTCATCACAAGTTTTAGG
PERK 3UTR-1	human	CGGGTAAATTAGGAATCTGC	GCTATTGAATGCTACAAATAG
PERK 3UTR-2	human	CAGTTTAATCATCTCACTTGC	AGTGTGTTCTGTACACCACC
PERK 3UTR-3	human	ATTCTCAGGCTGCAGAGGAG	GCTTTACGCTGGATGTTGC
IRE1del1 R		GAGGGGCCGCCCTCGCTCAGAGGGCGTCTGGAGTCA	
IRE1del1 F		GAGAGGTGGGGGATGCTGAGGAGGGGGAGGACGGAG	
IRE1del2 R		CTCCGTCCTCCCCCTCCTCAGCATCCCCCACCTCTC	
IRE1del2 F		CTCCTTCGTCCCCAAGGCCGGTGGAACAAGAGGCT	
DEL1 qPCR-1R		GAAATCTCACACTGCAGGACG	
DEL1 qPCR-2R		TCCCTAATGCCACACCTCATG	